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(57) Abstract

A double transdominant fusion gene (*trev*) to simultaneously inhibit two functions essential for HIV expression was constructed by linking *tat* and *rev* transdominant mutants. Trev was shown to independently inhibit both Tat and Rev functions in a human T cell line. Stably expressed Trev localized within the nucleus and exhibited a greater combinatorial effect than either single transdominant gene alone. Cells transfected with Trev showed a stable 20 to 30 fold inhibition of HIV propagation and were protected against viral cytopathic effects. Simultaneous inhibition of two essential viral genes present significant advantages for potential gere therapy treatment of HIV infection.

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A DOUBLE TRANSDOMINANT FUSION GENE AND PROTEIN

This invention was made with government suppport under Grant No. USPH5UOIAI30243, awarded by the National Institute of Health. The government has certain rights in this invention.

TECHNICAL FIELD OF THE INVENTION

The present invention relates generally to a double transdominant fusion gene (*trev*) and a method of treating HIV infection. More particularly, it relates to a combination of mutant *tat* and mutant *rev* genes to form a transdominant fusion gene.

BACKGROUND ART

"Intracellular immunization" is the concept that the introduction of an exogenous gene into cells will render such cells resistant to specific pathogens. Various strategies for intercellular immunization against HIV have been proposed, including antisense transcripts, ribozymes, suicide gene expression, RNA decoy expression and transdominant suppressors.

Tat and Rev are HIV-encoded regulatory proteins essential for efficient viral replication. Tat is a potent positive regulator which interacts with a stem loop RNA structure (TAR) located at the 5′ end of all HIV-1 transcripts. Tat is a small nuclear protein from which the

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first 67 amino acids are sufficient for transactivation. A conserved cysteine-rich region between amino acids 27 and 57 is important for protein-protein interactions and a basic region between amino acids 48 and 57 is required for nuclear localization and binding to TAR. Mutations in the latter have yielded potent transdominant suppressors. Rev is a 116-amino acid protein required for nuclear export of incompletely spliced transcripts necessary for HIV structural gene and full genomic expression. Rev contains an arginine-rich base dominant between amino acids 35 and 51 required for nucleolar localization and binding to the rev responsive element (RRE), and a leucine-rich dominant between amino acids 75 and 83 important for protein-protein interactions. Mutations in the protein-protein interaction domain have yielded effective transdominant suppressors of wild-type Rev function.

Previous studies report that the addition of a TAR decoy to a Rev transdominant vector enhanced significantly the inhibitory effect of the transdominant construct. Further, the construction of a retroviral vector which could express either a transdominant *tat* or a transdominant *rev* was significantly more effective than comparable vectors which expressed one transdominant and one wild-type *tat* or *rev*. These reports suggest possible benefits of inhibiting *tat* and *rev*.

Most proposed strategies attack a single stage of viral expression. The present invention describes a fusion protein which simultaneously inhibits both proteins. It includes a double transdominant molecule which simultaneously inhibits *tat* and *rev*, two essential viral proteins. The double transdominant has functional advantages over single inhibitors.

SUMMARY OF THE INVENTION

An object of the present invention is the provision of a double transdominant fusion gene.

An additional object of the present invention is the provision of a method for the treatment of HIV disease.

A further object of the present invention is the provision of a double transdominant fusion protein.

Thus, in accomplishing the foregoing objects there is provided in accordance with one aspect of the present invention, a double transdominant fusion gene, comprising: a *tat* transdominant mutant gene linked to a *rev* transdominant mutant gene, wherein said double transdominant fusion gene inhibits expression of HIV.

In a specific embodiment of the present invention, codons in the *tat* transdominant mutant gene which code for basic amino acids at positions 52 to 57 of the Tat protein are replaced with codons which code for neutral amino acids.

More specifically, the codon sequences for the amino acids arg, arg, gln, arg, arg and arg are replaced with the codon sequences for the amino acids gly, gly, ala, gly, gly and gly.

In an additional specific embodiment of the present invention, codons in the *rev* transdominant mutant gene which code for amino acids at positions 80 to 82 of the Rev protein have been deleted.

In a preferred embodiment, the *tat* and *rev* transdominant mutant genes are linked by a histidine bridge.

An additional embodiment of the present invention provides the protein encoded by the double transdominant fusion gene.

Another embodiment of the present invention includes a method of treating HIV disease in humans comprising delivering to the human to be treated a pharmacologically effective dose of a double transdominant gene containing a *tat* transdominant mutant gene linked to a *rev* transdominant mutant gene.

Other and further objects, features and advantages will be apparent and the invention will be understood more readily from a reading of the following specification and by reference to the

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accompanying drawings, forming a part thereof, where examples of the present preferred embodiments of the invention are given for purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration of the structure of a double *trev* transdominant fusion gene and its double transdominant fusion protein.

Figure 2 shows the comparison of inhibition of function by each single transdominant mutant genes (*tat* and *rev*) and the double transdominant gene (*trev*).

Figure 3 shows the effects of transdominant constructs on transient expression of proviral vectors.

Figure 4 shows a comparison of the effects of stable transdominant genes (*tat*, *rev*, *trev*) on HIV-1 challenge.

Figure 5 shows the results from a long-term cytopathicity protection assay.

Figure 6 shows the p24 results of HIV-1_{NL4-3} infection of 1G5 cells transduced with the S3 Trev retroviral vector.

Figure 7 shows the viable cell number results of HIV-1_{NL4-3} infection of 1G5 cells transduced with the S3 Trev retroviral vector.

Figure 8 shows the effects of transdominant constructs on transient expression of a proviral vector.

Figure 9 shows the effects of infection with HIV-1_{NL4-3} of stable transdominant cell lines.

Figure 10 shows the effects of long-term HIV mediate cylopathicity in 1G5 and 1G5-Trev cells.

Figure 11 demonstrates the protection conferred by Trev against HIV strains NL4 (A), SF2 (B), clinical isolate 301714 (C) and clinical isolate 301657 (D). Filled symbols represent Trev-transduced cell cultures. Open symbols represent non-transduced cell culture. The m.o.i. used were: 0.002 (Squares,■), 0.004 (triangles,▲)and 0.02 (diamonds,♦).

Figure 12 demonstrates the survival advantage conferred by Trev. Viable cells in culture were counted every 5 days after infection number were expressed as percentage of non-infected cell counts cultured in parallel under the same conditions. A. HIV strain SF2. B. HIV Clinical isolate 301657. Open symbols represent non-transduced cell culture. The m.o.i. used were: 0.002 (Squares,■), 0.004 (triangles,▲)and 0.02 (diamonds,♦).

Figure 13 Trev does not induce resistant viral mutants. Supernatants taken from Trev transduced cell cultures after 30 days of HIV virus ('recovered') or a 1000-fold dilution of stock HIV virus ('stock'), were used to infect primary $CD4^+$ T cells at a m.o.i. of 0.002. HIV p24 Ag was measured 20 days after infection.

The drawings and figures are not necessarily to scale and certain features of the invention may be exaggerated in scale as shown in schematic form in the interest of clarity and conciseness.

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DETAILED DESCRIPTION OF INVENTION

It should be apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

As used herein, the term "vector" refers to the means by which the transdominant mutant *trev* gene can be introduced into a host organism or a tissue. There are various types of vectors, including plasmids, bacteriophages and cosmids.

As used herein, the term "Tat" refers to an HIV-coded regulatory protein essential for efficient viral replication. *Tat* is a potent positive regulator which acts on a stem loop RNA structure (TAR) located at the 5' end of all HIV-1 transcripts. Tat is a small 86 amino acid nuclear protein from which the first 67 amino acids are sufficient for transactivation. A conserved cysteine-rich region between amino acids 27 to 37 is important for protein-protein interactions. A basic region between amino acids 48 to 57 is required for nuclear localization and binding to TAR. A schematic representation of Tat is shown in Figure 1.

As used herein, the term "*tat*" refers to the gene which encodes Tat.

As used herein, the term Rev refers to a HIV coded regulatory protein essential for efficient viral replication. Rev is a 116-amino acid protein required for nuclear export of incompletely spliced transcripts necessary for HIV structural gene and full genome expression. Rev contains an arginine-rich basic domain between amino acids 35 to 51, which is required for nucleolar localization and binding to the rev responsive element (RRE), and a leucine rich domain between amino acids 75 to 83 which is important for protein-protein interactions. A schematic representation of Rev is shown in Figure 1.

As used herein the term "*rev*" refers to the gene which encodes Rev.

As used herein, the term "Trev" refers to a protein containing transdominant portions of both a Tat and Rev, which portions have noncompeting modes of action. In Trev, the basic amino acids at positions 52 to 57 of the Tat portion have been replaced with neutral codons and the Rev portion contains a three-amino acid deletion at positions 80 to 82. The Tat and rev portions of Trev are connected by a histidine bridge. A schematic representation of Trev is shown in **Figure 1**.

As used herein, the term "*trev*" refers to a double transdominant mutant gene comprised of a combination of at least one *tat* and at least one *rev* transdominant mutant gene.

One embodiment of the present invention is a double transdominant fusion gene comprising a *tat* transdominant mutant gene linked to a *rev* transdominant mutant gene. The fusion gene, *trev*, is designed to express complete Tat and Rev transdominant proteins with non-competing modes of action. The double transdominant fusion gene will inhibit the expression of HIV.

In the preferred embodiment, the *tat* transdominant mutant gene has the codons which code for the basic amino acids at position 52 to 57 of the Tat protein replaced with the codons which code for neutral amino acids. One skilled in the art recognizes readily that a variety of substitutions are available.

In the preferred embodiment of the present invention, the codons for the Tat amino acid sequence arg, arg, gln, arg, arg and arg have been replaced with the codon sequence for the amino acids gly, gly, ala, gly, gly and gly.

In the preferred embodiment of the present invention, the *rev* transdominant mutant gene has the codons which code for amino acids at positions 80 to 82 of the *rev* protein deleted.

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In the preferred invention, the *tat* and *rev* transdominant mutant genes are linked by a histidine bridge.

Another embodiment of the present invention includes a method for treating HIV disease in humans comprising delivering to the human being treated a pharmacological dose of a double transdominant gene containing a *tat* transdominant mutant gene linked to a *rev* transdominant mutant gene.

The composition of the present invention can be formulated according to known methods to prepare pharmacologically useful compositions. The compositions of the present invention or their functional derivatives are combined in admixture with a pharmacologically acceptable carrier vehicle. Suitable vehicles and their formulations are well known in the art. In order to form a pharmacologically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the double transdominant fusion gene or its equivalent or the functional derivative thereof, together with the suitable amount of carrier vehicle.

The composition of the present invention will usually be formulated in a vector. The vector can be administered by a variety of methods including parenterally, by injection, rapid infusion, nasopharyngeal absorption, dermal absorption or orally. The compositions may alternatively be administered intramuscularly or intravenously. In addition, the vector for parenteral administration can further include sterile aqueous or nonaqueous solutions, suspensions and emulsions. Examples of known nonaqueous solvents include propylene glycol, polyethylene glycol, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Carriers, adjuncts or occlusive dressings can be used to increase tissue permeability and enhance absorption. Liquid dosage forms for oral administration may generally comprise a liposome solution. Suitable forms for suspension

include emulsions, solutions, syrups and elixirs containing inert diluents commonly used in the art, such as purified water. Besides the inert dilutants, such compositions can also include wetting agents, emulsifying and suspending agents or sweetening, flavoring, coloring or perfuming agents.

Additionally, pharmaceutical methods may be employed to control the duration of action. These are well known in the art and include control release preparations and can include appropriate macromolecules, for example polymers, polyesters, polyamino acids, polyvinyl, pyrolidone, ethylenevinylactate, methylcellulose, caroboxymethylcellulose, or protamine sulfate. The concentration of macromolecules, as well as the methods of incorporation, can be adjusted in order to control release. Additionally, the vector could be incorporated into particles of polymeric materials such as polyesters, polyamino acids, hydrogells, poly (lactic acid) or ethylene vinylacetate co-polymers. In addition to being incorporated, these agents can also be used to trap the vectors in microcapsules. These techniques are known in the art.

A composition is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient patient. Such an agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in detectable change in the physiology of a recipient patient.

Generally, the dosage needed to provide an effective amount of composition will vary depending on such factors as the recipient's age, condition, sex and extent of disease, if any, and other variables which can be adjusted by one of ordinary skill in the art.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner.

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Example 1

Plasmid Construction

The fusion gene, *trev*, was designed to express complete Tat and Rev transdominant proteins with noncompeting modes of action (**Figure 1**). Generally, in trev the basic amino acids at positions 52 to 57 of Tat were substituted for neutral codons (*tat 52/57*) and joined by a histidine bridge to a three amino acid deletion at positions 80 to 82 of Rev (*rev*Δ80-82). Both sequence changes have independently been shown to generate potent negative single transdominants.

The mode of action of Tat 52/57 is not known but does not involve competition for TAR binding nor inhibition of wild type Tat from nuclear localization and is thought to function by competition for cellular factors. Rev Δ80-82 localizes to the nucleus and is thought to function by binding to RRE but not to necessary cellular factors. Thus, in Trev the Tat portion may be competing with wild type Tat for soluble cellular factors while the Rev portion is competing with wild type Rev for RRE binding. These two modes of action should not interfere with each other. A fusion construct was chosen to assure simultaneous expression of both functions and allow greater versatility of choices for delivery systems.

The specific construction of Trev is depicted in Figure 1. The first 72 amino acids from HIV 1-Tat with the indicated substitutions were amplified by PCR from the Tat transdominant gene, *tat* 52 - 57 using 5′-CGCGCATATGGCAGGAAGAAGCGGAG-3′ as a 5′ primer and 5′-CTAACAGATCTATTCTTTAGCTCCTGACTCCAA-3′ as a 3′ primer. The amplification product was cloned into the *Hinc*II site of pBluescript KS (Stratagene, La Jolla, CA, USA) and sequenced to verify the presence of the desired substitutions. An *Nde*I site was created at the 3′ end of *tat* 52 - 57 for in-frame insertion of *rev*. The 80 - 82 deletion in the Rev coding sequence was obtained as an *Nde*I-*Eco*RI

fragment in pBR322 and subcloned into *Nde*I-*Eco*RI of the above construct. The final product was sequenced to verify transdominant mutations and in-frame open reading sequences for both *tat* and *rev* portions. An *Rsa*I-*Spe*I fragment containing the *trev* sequence was substituted for Bgal into the *Sma*I-*Xba*I site of pPGK-Bgal. The PGK (phosphoglycerate kinase) promoter has been shown to provide efficient expression in the hematopoietic cell lineage. The Tat 52 - 57 expression vector was pDex (RSV-LTR, *tat* 52/57, SV-40 poly A site). The Rev M10 expression vector was pBC12-M10 (cytomegalovirus (CMV)-immediate-early promoter, *Rev-M10*, rat pre-pro insulin intron-poly A site).

pRLR was constructed by cloning a 2.2-kb fragment containing an RSV-LTR promoter and the firefly luciferase coding sequence without a poly-adenylation signal 5′ to a 4.4-kb fragment of HIV-1 containing most of the *env* sequence, the RRE, and the 3′ LTR and poly A signal in a pBluescript backbone.

pS3Trev was constructed by subcloning a 1450-bp *Sal*I to *Not*I PGK-trev fragment from pPGK-*trev* into the *Sal*I and *Stu*I sites in the body of the pS3 retroviral vector backbone.

Example 2

Cell Culture and Analysis

Cells were maintained in RPMI 1649 (GIBCO BRL, Gaitherburg, MD USA) with 10% calf serum (HyClone, Logan, UT, USA) at densities of less than 10^6 cells per millimeter. All transfections were carried out in triplicate by electroporation in 125 µl of growth medium, 125 V, 3000 µF in a BTX-ECM600 electroporator. The total amount of DNA per transfection was adjusted to 20 µg using pUC-12 as carrier. Unless otherwise indicated, the concentrations were 1 µg per transfection of effector plasmid (wild-type *tat* or HIV-1) and 5 µg per transfection of the transdominant gene plasmid. Luciferase analyses were performed

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16-24 h after transfection using the Luciferase Analysis System (Promega, Madison WI, USA), as per the recommendation of the manufacturer. Cell viability was measured by trypan blue exclusion and p24 antigen was measured by ELISA from 50 µl of cell supernatant (Coulter).

In **Figures 2, 3 and 8**, the Tat analyses were done by cotransfection of wild type Tat with the transdominant plasmid in 1:5 molar ratio into the reporter cell line 1G5. The positive control shows the effects of wild type Tat without a transdominant, the negative control shows the effect of carrier DNA alone. Rev transdominant analyses were done by cotransfection of pRLR (1 µg) with wild type Rev (1 µg) and the transdominant plasmid in a 1:5 molar ratio into Jurkat cells. In **Figure 8**, the proviral clone HIV-1_{NL4-3} was cotransfected into 1G5 reporter cells.

In **Figures 4 and 9**, triplicates of $2.5x10^5$ parental (1G5) and stably transfected cells were seeded in six well plates. The cells were all infected with equal aliquots of a low titer HIV-1_{NL4-3} virus stock on day 1. Every three days $2.5x10^5$ cells were harvested from each well for luciferase activity analysis and $2.5x10^5$ cells were re-seeded in fresh media for subsequent analysis.

In **Figures 5 and 10**, two six-well dishes each with triplicates of $2.5x10^5$ parental (1G5) and 1G5-Trev cells were seeded at day 0. One set of triplicates was infected with equal aliquots of a low titer HIV-1_{NL4-3} virus stock and the second set was used as control. Every seven days (14 days for last data point) the cultures were split 1:4. 75% of the cells were removed and counted by Trypan Blue exclusion, 25% were used to maintain the culture.

HIV infection was done by direct addition of viral supernatant to the growth medium at an approximate multiplicity of infection of 0.001. Tat retroviral vector transduction was done by exposure of the cells to the viral supernatant in growth medium supplemented with Polybrene

to 4 μg/ml. After overnight incubation the cells were changed to normal growth medium.

Example 3

Immunocytochemistry

Cytospin preparations were fixed with cold methanol for 3 min, blocked with a 10% solution of normal goal serum and incubated with a 1:800 dilution of rabbit anti-Rev primary antibody (Art 27/51) overnight at 4°C[20]. Detection was accomplished using the suggested super-sensitive conditions from a StrAviGen MultiLink kit (Biogenex, San Ramon, CA, USA) followed by stable DAB from Research Genetics (Huntsville, AL, USA) at room temperature for 3 min and a hematoxylin counterstain.

Example 4

Cotransfection

Trev was evaluated for lack of Tat or Rev transactivating activity and independent Tat and Rev transdominant activity. In cotransfection experiments with Tat dependent and Rev dependent luciferase constructs, Trev showed no transactivation potential.

A. Tat Transdominant Activity. Tat transdominant activity was evaluated by cotransfection with wild type Tat into the reporter cell line 1G5. These cells contain an integrated HIV long terminal repeat (HIV^{LTR}) luciferase construct highly sensitive to Tat transactivation. Trev showed approximately an eight fold inhibition of Tat transactivation compared to about 30 fold observed with the single transdominant Tat 52/57 (**Figure 2**). Comparable inhibition results were obtained with a transient Tat-dependent HIV^{LTR} luciferase construct in cells derived from the human Jurkat T-cell line. Trev was also effective on a 1G5 clone with stable constitutive Tat expression.

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B. **Rev Transdominant Activity.** Rev transdominant activity was analyzed by cotransfection of a Rev dependent luciferase construct with wild type Rev and Trev into Jurkat cells (pRLR). Trev inhibited Rev transactivation by up to 10 fold, comparable to the inhibition observed with an equivalent single transdominant, M10 (**Figure 2**). The mechanism for the observed near two fold inhibition by M10 in the Tat assay is not evident, but probably reflects nonspecific plasmid effects. However, M10 was not inhibitory, but rather stimulated Tat effects when a full proviral HIV construct was used. Only the fusion protein Trev was efficient in independently inhibiting both Tat and Rev function.

C. **Simultaneous Tat and Rev Activity.** Simultaneous inhibition of Tat and Rev was analyzed by cotransfection with a provirus clone of HIV-1_{NL4-3} and infection of stably transfected cells with HIV-1_{NL4-3} virions. Cell viability was measured to evaluate protection against cytopathic effects, luciferase was analyzed as an indicator of Tat activity and p24 was used as an indicator of viral particle production. The single transdominant Tat 52/57 was most efficient in transient transfection assays, closely followed by Trev (see **Figures 3 and 8**). The single Rev transdominant M10, however, showed a surprising two fold increase in luciferase activity; although it did protect against cytopathic effects and viral particle production. The increase in luciferase activity promoted by M10 may reflect an increased production of multiply spliced HIV transcripts (therefore Tat) as a result of the inability to export incompletely spliced transcripts from the nucleus. This would create a potent positive feedback loop.

To analyze further the effects of coordinate Tat and Rev inhibition the double and single transdominants were stably expressed in 1G5 cells. Populations of transfected cells were studied to minimize possible effects of clonal variation on transdominant gene expression or susceptibility to HIV infection. Expression of transdominants in the

stable lines was analyzed by transient inhibition assays of Tat transactivation in Tat 52/57 and Trev transfected cells and of Rev in M10 and Trev transfected cells. All stable cell lines retained their relative transdominant activities and there were no detectable deleterious effects on the growth characteristics of the transfected cell populations. Tat 52/57 localized to both the cytoplasm and nucleus and RevΔ80-82 was shown to localize to the nucleus. In the stably transfected cells, Trev protein was shown to localize to the nucleus by immunocytochemistry, indicating that Trev retained a structure for appropriate subcellular localization.

Stable transfected cell populations were then challenged with infectious HIV-1_{NL4-3}. All three transdominant genes showed significant suppressor effects four days after HIV infection. However, suppression by Tat 52/57 was short in duration and approached control levels between seven and 10 days after infection (**Figures 4 and 9**). Inhibition by M10 reached a plateau at approximately 50% inhibition for up to ten days. Trev transfected cells showed a 75% inhibition for the same length of time (**Figures 4 and 9**).

To confirm the inhibition observed was not a result of undetected, down-regulation of CD4 expression, 1G5 parental and 1G5-Trev cells were transduced with an amphotropic murine leukemia virus (MuLV) based Tat retroviral vector which does not require CD4 for infection. 1G5-Trev cells showed a 50-60% inhibition compared to the parental cell line. 1G5-Trev cells were also compared with 1G5 parental cells on a long term cell survival and luciferase activity assay. Trev showed continuous long term suppression of luciferase activity and a significant reduction of the cytopathic effects of HIV infection for the 63 day duration of the experiment (**Figures 5 and 10**).

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Example 5

Gene Therapy

It is expected that 74% of currently HIV-infected people will develop the acquired immunodeficiency syndrome (AIDS) in the next 5 years. Unfortunately, anti-HIV therapy is far from optimal; it improves quality of life delaying the onset of AIDS, but does not significantly reduce mortality. In the last few years, gene therapy has been suggested as an alternative treatment for HIV infection. A therapeutic gene can be transduced into mature $CD4^+$ T cells or into pluripotent hematopoietic stem cells to generate protected $CD4^+$ T cells and monocytes, the primary targets of HIV infection. Cell populations resistant to HIV replication might decrease viral spread and allow survival of cells resistant to the cytopathicity induced by the virus.

Gene therapy is one of several approaches that are being tested in the search for an effective anti-HIV treatment. In this strategy a "protective" gene is introduced into target cells, rendering them relatively resistant to the viral cytopathicity. Experiments in primary cells are necessary to exclude the possibility of a special condition in cell lines that could be responsible for the inhibition of viral replication. Primary cell lines resemble more closely *in vivo* conditions, and are an art-accepted model for gene therapy experimentation. *Trev* was transduced to primary $CD4^+$ T lymphocytes from different donors, and then the lymphocytes were infected with different HIV strains. Trev expression was shown to be localized to the nucleus, confirming the data with Jurkat cells.

In addition, T cell growth and CD4/CD8 expression was studied to identify possible effects of Trev transduction in normal cell function. These parameters were similar before and transduction, suggesting that Trev may not alter cell functions. This result corroborates similar studies using other anti-HIV therapeutic genes. See Malim, et al., *J. Exp. Med.* 176:1197-12a; Gunther, et al., *Hum. Gene Ther.* 4:643-645 (1993)

The potential to induce escape viral mutants is an important consideration in any HIV treatment strategy. This issue was addressed by taking supernatants from the last cultures and infecting new Trev-transduced cells. If the surviving virus is pathogenic and overcomes Trev effect, no protection would have been seen. This evidence plus the fact that Trev protects cells for a long period of time suggests that escape mutants may not be induced.

The feasibility of protecting primary T cells against different HIV strains, by transduction with Trev is an important step for the development of gene therapy for HIV infection. Trev is a unique transdominant protein, inhibiting both Tat and Rev activities simultaneously. This effect may be important considering that inhibition of Rev favors the expression of the two-exon sequence of *tat*. This protein is responsible for a variety of effects different from the activation of the HIV-LTR promoter, which may contribute to the pathogenesis of AIDS.

Methods

A. **Cell cultures.** Peripheral blood leucocyte fractions were obtained from the Gulf Coast Regional Blood Center (Houston, Texas). Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. Cells were maintained in RPMI 1640 (Sigma) media supplemented with 10% FCS (Hyclone) and 50 U/ml IL-2 (R&D). Mononuclear cells were stimulated to proliferate with 4 μg/ml phytohemagglutinin (PHA) (Sigma) for three days.

B. **Gene transduction and CD4 selection.** Amphotropic retroviral vectors carrying Trev are produced by the packaging cell line AMT9. 10^7 PHA stimulated mononuclear cells were transduced by coculture in a $75cm^2$ flask (Costar) with irradiated (60Gy) confluent AMT9 cells for 2 days in RPMI 1640 with 10% FCS, 50 U/ml IL-2 and 4 μg/ml Polybrene (Gibco). After the transduction procedure, cells in

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suspension were collected and CD4$^+$ T cells were sorted by flow cytometry (Coulter).

C. **HIV infections.** Primary CD4$^+$ T cells were cultured in RPMI 1640 supplemented with 10% FCS and 50 U/ml IL-2. Laboratory (NL-4, SF2) or community (clinical isolates 301714, 301657) strains of HIV (AIDS reagent program) were added to the cultures at three different multiplicity of infection (m.o.i): 0.002, 0.004 and 0.02. The following day, culture media was replaced with fresh media. Samples were taken every 5 days. Cells were maintained below 10^6 cell/ml, splitting cell cultures when they reached that concentration. Cell viability was measured by Trypan Blue exclusion and p24 Antigen present in supernatants was measured by ELISA (Coulter).

D. **Western Blot.** Transduced and non-transduced CD4$^+$ T cells were harvested for protein extraction. Cells were lysed with a low salt buffer and a nuclear pellet was obtained. Nuclei were lysed with a high-salt buffer to obtain nuclear protein extracts (Dignam, et al., *NAR* II: 1475-1489 (1983)). Nuclear protein extracts were separated by electrophoresis in a 15% SDS-polyarylamide gel. The gel was transferred to a nitrocellulose membrane (Costar). This membrane was blocked with BLOTTO for 20 minutes, washed and incubated with anti-Tat polyclonal antibody overnight at 4°C. An anti-rabbit IgG coupled and alkaline phosphatase (Sigma) was incubated for one hour at room temperature. NBIP/NBT reagent (Amersham) was used for detecting signal.

E. **Immunocytochemistry.** Cells were fixed in coverslips with cold methanol, blocked with BLOTTO for 30 minutes at room temperature and incubated with rabbit polyclonal anti-Tat antibody overnight at 4°C. The Vectastain peroxidase-based kit (Vector) was used for detection, according to manufacturer's recommendations.

F. **HeLA betaβ-gal Assay.** HeLaβgal cells (Kimpton, J. and M. Emerman, *J. Virol.* 66:2232-2239 (1992)) reaching 30% confluence were

plated in 24-well tissue-culture dish and infected with 10-fold dilutions of virus-containing supernatants. After 48 hrs of incubation at 37°C, cells were washed with PBS and fixed with a 1% formaldehyde solution for 5 minutes. After a new wash with PBS, 200 µl of staining solution (4mM $K_3Fe(CN)_6$, 4mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 400 mg/ml X-gal (Gibco)) were added and incubated at 37°C for 1 hr. The HeLaβgal cells can be infected by HIV and carry the b-galatosidase gene driven by the HIV-LTR promoter. Nuclei of infected cells stain blue. Virus titer was determined by the greatest dilution where blue cell nuclei were observed.

Results

A. **Trev expression in transduced PBLs.** PBLs obtained from normal healthy donors were stimulated with PHA for 72 hr and transduced with a retroviral vector carrying the *trev* gene. Trev expression in transduced cells was confirmed by immunocytochemistry. Trev protein was localized to the nucleus and was detectable in 50-60% of the cells. Trev protein was also detected in Western blots of cell nuclear lysates, with an approximate molecular weight of 34kD.

Growth curves of transduced cells and non-transduced cells, assessed by counting viable cells using Trypan Blue exclusion, were similar. CD4/CD8 ratios were evaluated by flow cytometry before and after Trev transduction, and no differences were detected.

B. **Protection assays.** Transduced and non-transduced cells were sorted for CD4 expression and challenged with four different HIV stains. Levels of p24 Ag in the cultures were measured to indicate HIV replication. Trev inhibited synthesis of p24 Ag in all experiments, using laboratory and community strains of HIV and different m.o.i. Trev-transduced $CD4^+$ T cells cultures infected with HIV laboratory strains NL-4 and SF2 produced no detectable p24 Ag, while control

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non-transduced cell cultures showed levels up to 5 ng/ml (**Figure 11 A & B**). Reduction of p24 Ag levels was also significant in transduced cell cultures infected with clinical isolates of HIV (**Figure 11 C & D**). Experiments also demonstrated that the degree of protection correlates with lower m.o.i. No detectable p24 Ag was found in cultures of Trev-transduced cells infected with three of the four virus strains at a m.o.i. of 0.002. With higher m.o.i., p24 Ag concentrations of less than 2 ng/ml were observed in the Trev-transduced cell cultures, compared to 8 to 10 ng/ml for non-transduced cell cultures. Using HelaCD4β-gal cells as indicators, infections viral particles in samples from Trev cultures were not detected, while supernatants from non-protected cells gave viral titers of 10^3 viral particles/ml (**Table 1**).

TABLE 1: HIV virus titration assay, using the HeLaβgal assay.*

Viral titers:

HIV strain	Trev	Control
Strain NL4	0	10^3
Strain SF2	0	10^2
Clin. Isolate 301714	0	10^2
Clin. Isolate 301657	0	10^3

*Supernatants from Trev-transduced lymphocyte cultures (trev) and non-transduced lymphocyte cultures (control) infected with different strains of HIV were assayed for viral titers using the HeLaβgal assay (See Material and methods) at day 20 after infection.

C. **Trev protected cells have a survival advantage.** Cell survival after HIV infection was estimated as a percentage of non infected cells carried in culture over the same period. Trev-transduced cells infected with HIV survive for a period similar to non-infected cells and longer than non-transduced cells. Cell death was observed in non-transduced cells by day 20, and cell counts dropped to below 50% of control non-infected cells counts by day 30. (**Figure 12**)

D. **Trev protection does not induce escape viral mutants.** To define if this strategy could induce escape viral mutants that are able to replicate in the presence of Trev, 1×10^5 Trev-transduced $CD4^+$ T cells were infected with non-diluted supernatants recovered from the 30-day collection time of HIV-infected Trev-transduced cultures. Additionally, 1×10^5 of non-transduced cells were infected with the same supernatants as controls. Although infective viral particles could not be found in these supernatants using the HeLAβgal assay, the supernatants contain enough viral particles to replicate in a new cell culture. After 20 days, levels of p24 Ag were 10-fold higher in non-transduced cell cultures than in Trev-transduced cell cultures, reproducing the results of previous experiments. Similar results were obtained with two different HIV strains: NL4 and clinical isolate 301657. If viruses would have mutated to overcome Trev protection, reduced inhibition of viral replication would be seen. Results showed that replication of virus obtained from Trev-protected cultures can still be inhibited by Trev, suggesting that Trev does not induce selection. (**Figure 13**).

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantage mentioned, as well as those inherent therein. The nucleotides, proteins, peptides, methods, procedures and techniques described herein are presently representative of the preferred embodiments, are intended to be exemplary and are not intended as limitations on the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of attached claims.

ISDOCID: <WO___9628189A1_I_>

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Belmont, John W.
Harper, J. Wade
Rice, Andrew P.
Butel, Janet S.
Chinen, Javier

(ii) TITLE OF INVENTION: Double Transdominant Fusion Gene and
Protein

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Fulbright & Jaworski L.L.P.
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
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(A) APPLICATION NUMBER: US 08/399,264

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(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Paul Esq., Thomas D.

(B) REGISTRATION NUMBER: 32,714

(C) REFERENCE/DOCKET NUMBER: D-5670

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 713 651-5151

(B) TELEFAX: 713 651-5246

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:

(C) UNITS: 26 bp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGCGCATATG GCAGGAAGAA GCGGAG

26

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(viii) POSITION IN GENOME:
(C) UNITS: 33 bp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTAACAGATC TATTCTTTAG CTCCTGACTC CAA

33

What is claimed is:

1. A double transdominant fusion gene, comprising:

a *tat* transdominant mutant gene linked to a *rev* transdominant mutant gene, wherein said double transdominant fusion gene inhibits expression of HIV.

2. The double transdominant fusion gene of claim 1, wherein codons in the *tat* mutant which code for basic amino acids at positions 52 to 57 of the Tat protein are replaced with codons which code for neutral amino acids.

3. The fusion gene of claim 2, herein a coding sequence for amino acids arg, arg, gln, arg, arg and arg is replaced with a coding sequence for amino acids gly, gly, ala, gly, gly and gly.

4. The fusion gene of claim 1, wherein codons of the *rev* mutant gene which code for amino acids at positions 80 to 82 of the Rev protein have been deleted.

5. The fusion gene of claim 1, wherein the *tat* and *rev* transdominant mutant genes are linked by a histidine bridge.

6. A double transdominant fusion gene, comprising:

a *tat* transdominant mutant gene, wherein codons of said *tat* mutant gene which code for basic amino acids at positions 52 to 57 of the Tat protein are replaced with codons which code for neutral amino acids;

a *rev* transdominant mutant gene, wherein the codons of the Rev mutant which code for amino acids at positions 80 to 82 of the *Tat* protein have been deleted; and

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a histidine bridge liking the *tat* transdominant mutant gene to the *rev* mutant gene.

7. The transdominant protein produced by the transdominant fusion gene of claim 6.

8. A method of treating HIV disease in humans, comprising:

delivering to the human to be treated a pharmacologically effective dose of a double transdominant gene containing a *tat* transdominant mutant gene linked to a *rev* transdominant mutant gene.

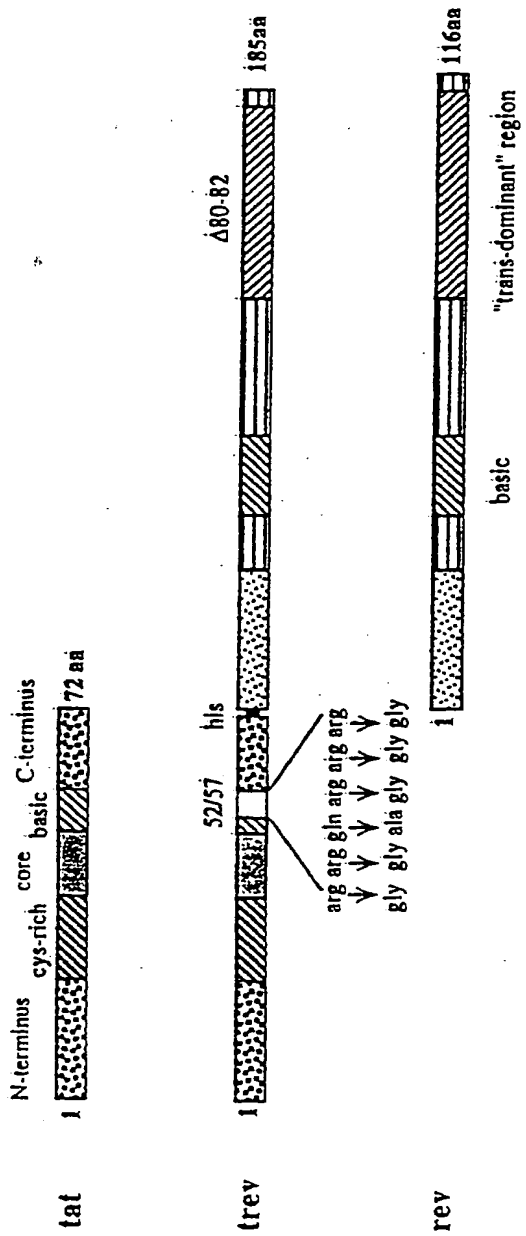

FIGURE 1

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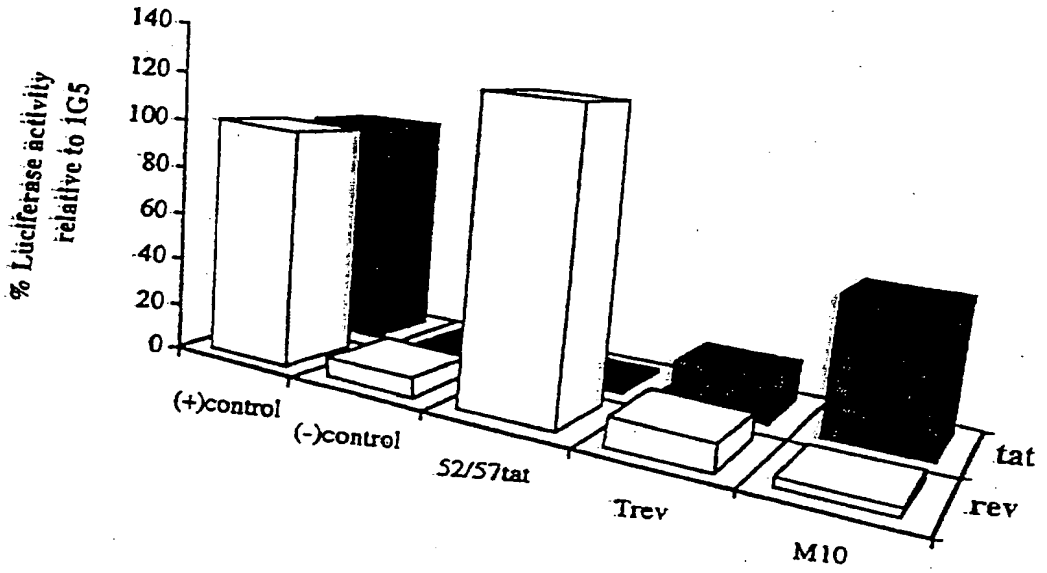
% Luciferase activity relative to IG5
140
120
100
80
60
40
20
0
(+)control
(-)control
52/57tat
Trev
M10
tat
rev

FIGURE 2

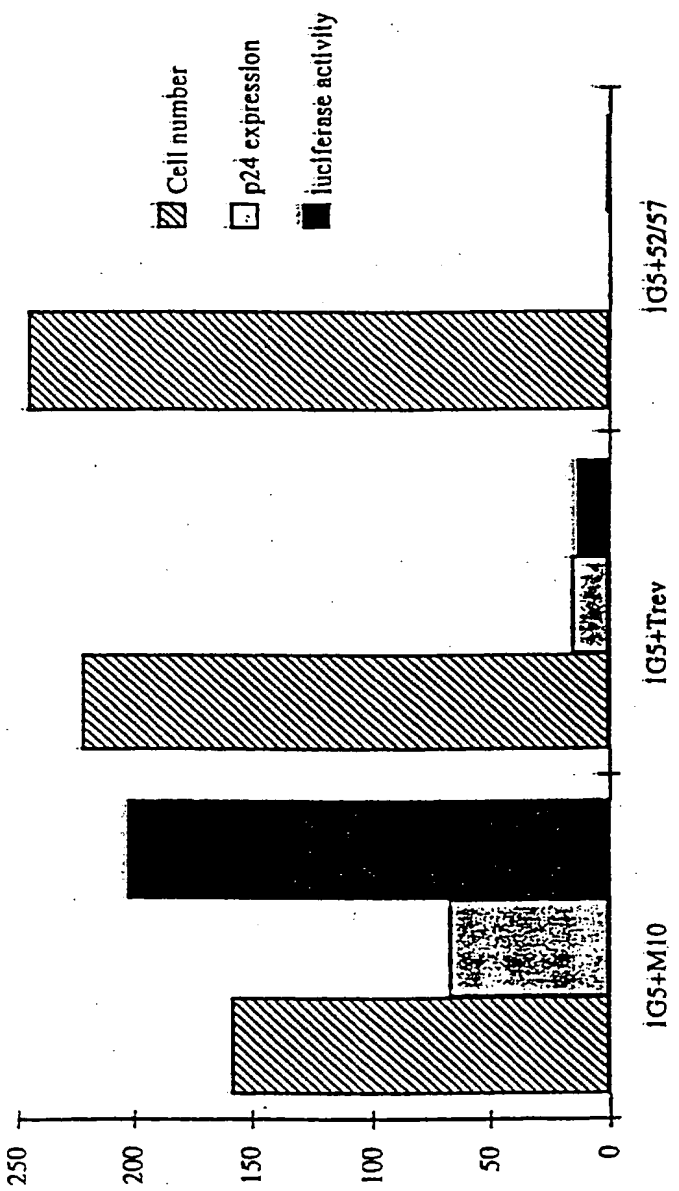

FIGURE 3

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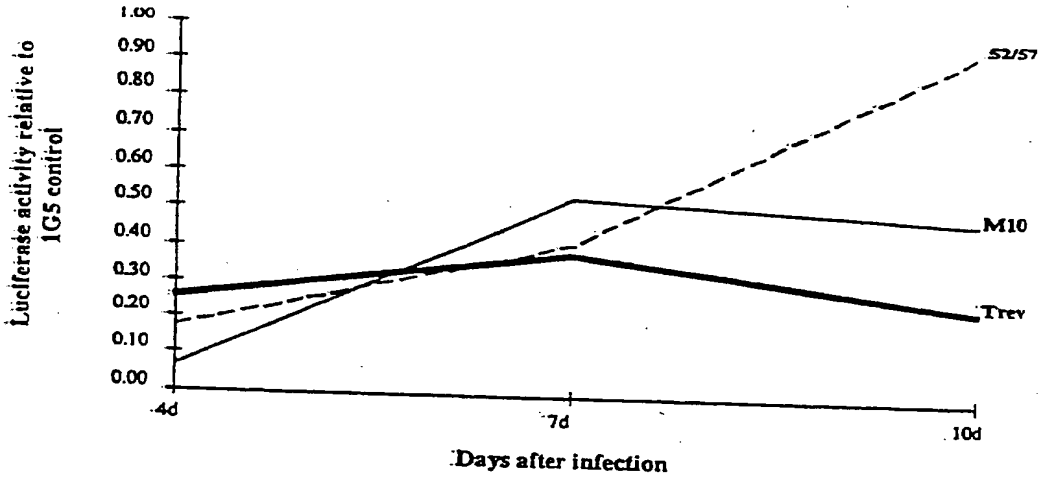
Luciferase activity relative to 1G5 control
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
-4d
7d
10d
Days after infection
S2/57
M10
Trev

FIGURE 4

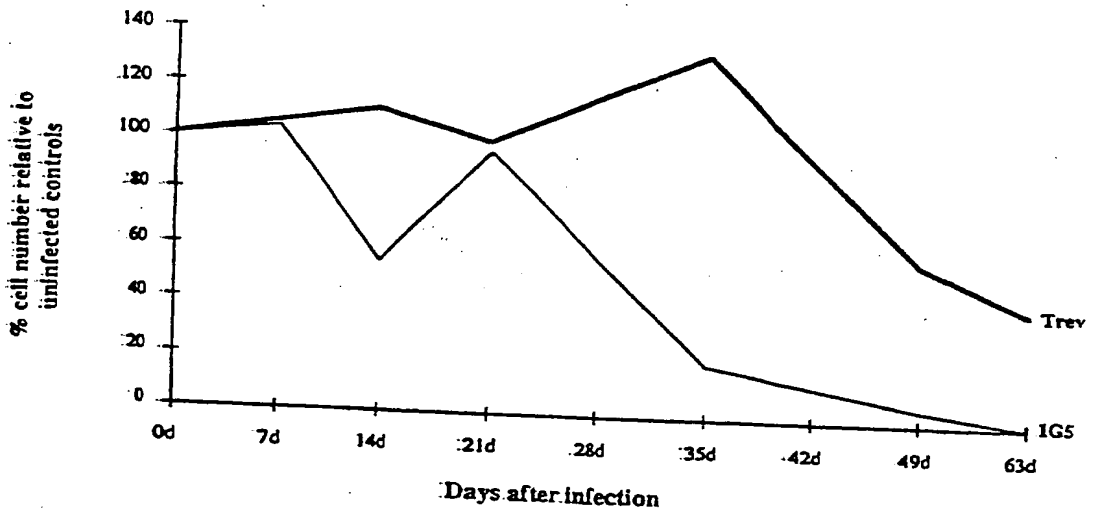

FIGURE 5

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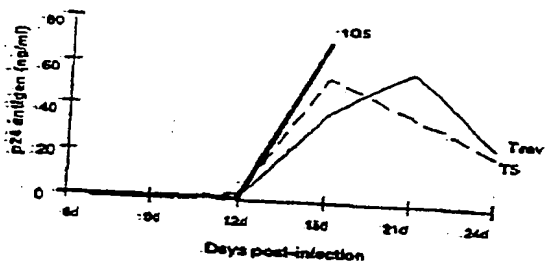
p24 antigen (ng/ml)
80
60
40
20
0
6d
9d
12d
15d
21d
24d
Days post-infection
105
Tsav
TS

FIGURE 6

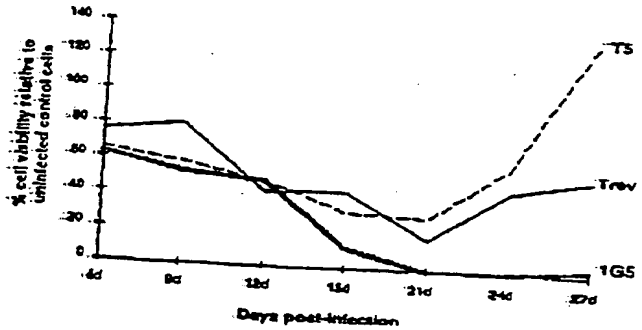

FIGURE 7

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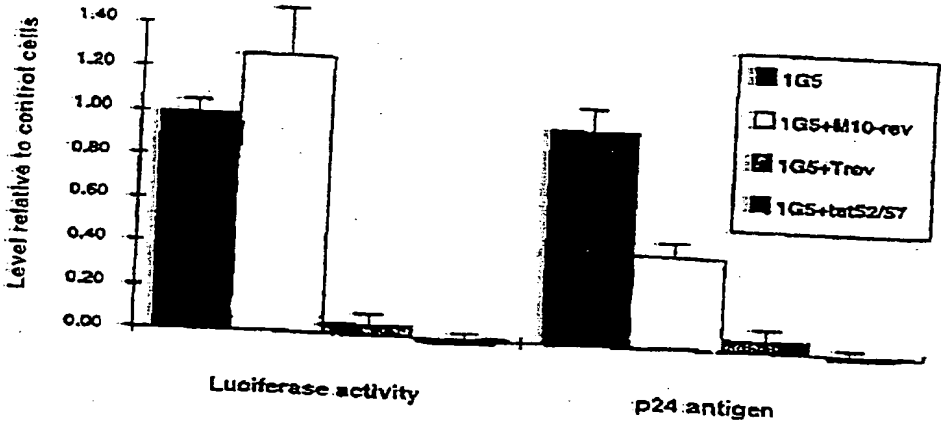
Level relative to control cells
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1G5
1G5+M10-rev
1G5+Trev
1G5+tat52/57
Luciferase activity
p24 antigen

FIGURE 8

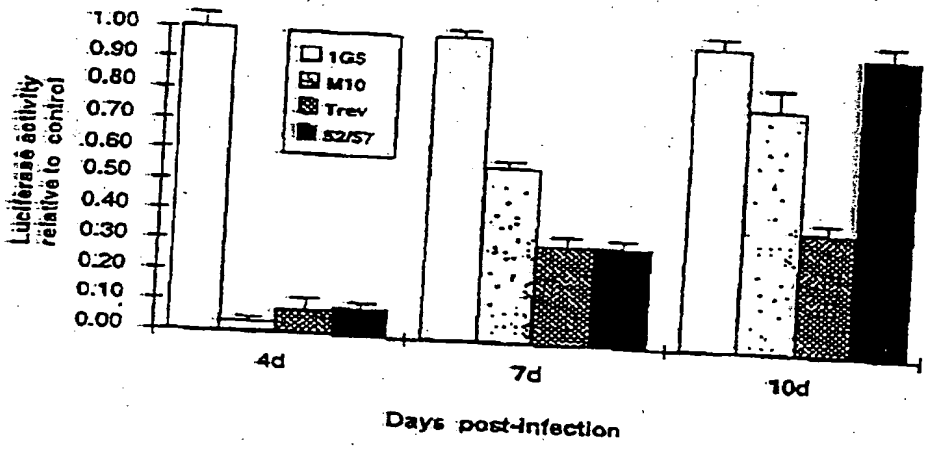

FIGURE 9

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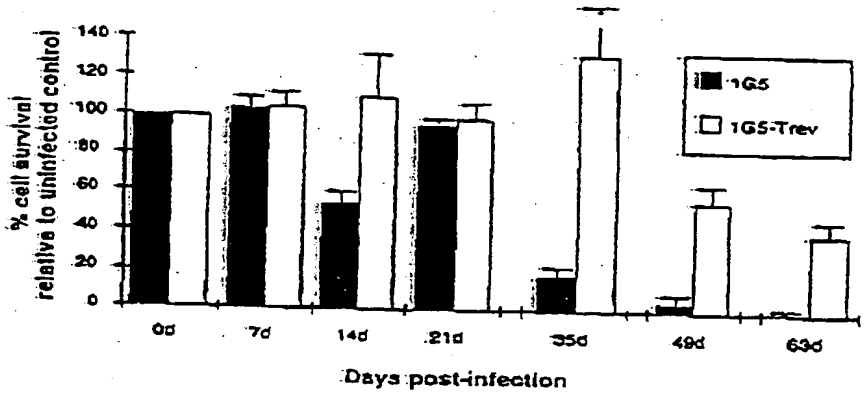
% cell survival relative to uninfected control
140
120
100
80
60
40
20
0
0d
7d
14d
21d
35d
49d
63d
Days post-infection
1G5
1G5-Trev

FIGURE 10

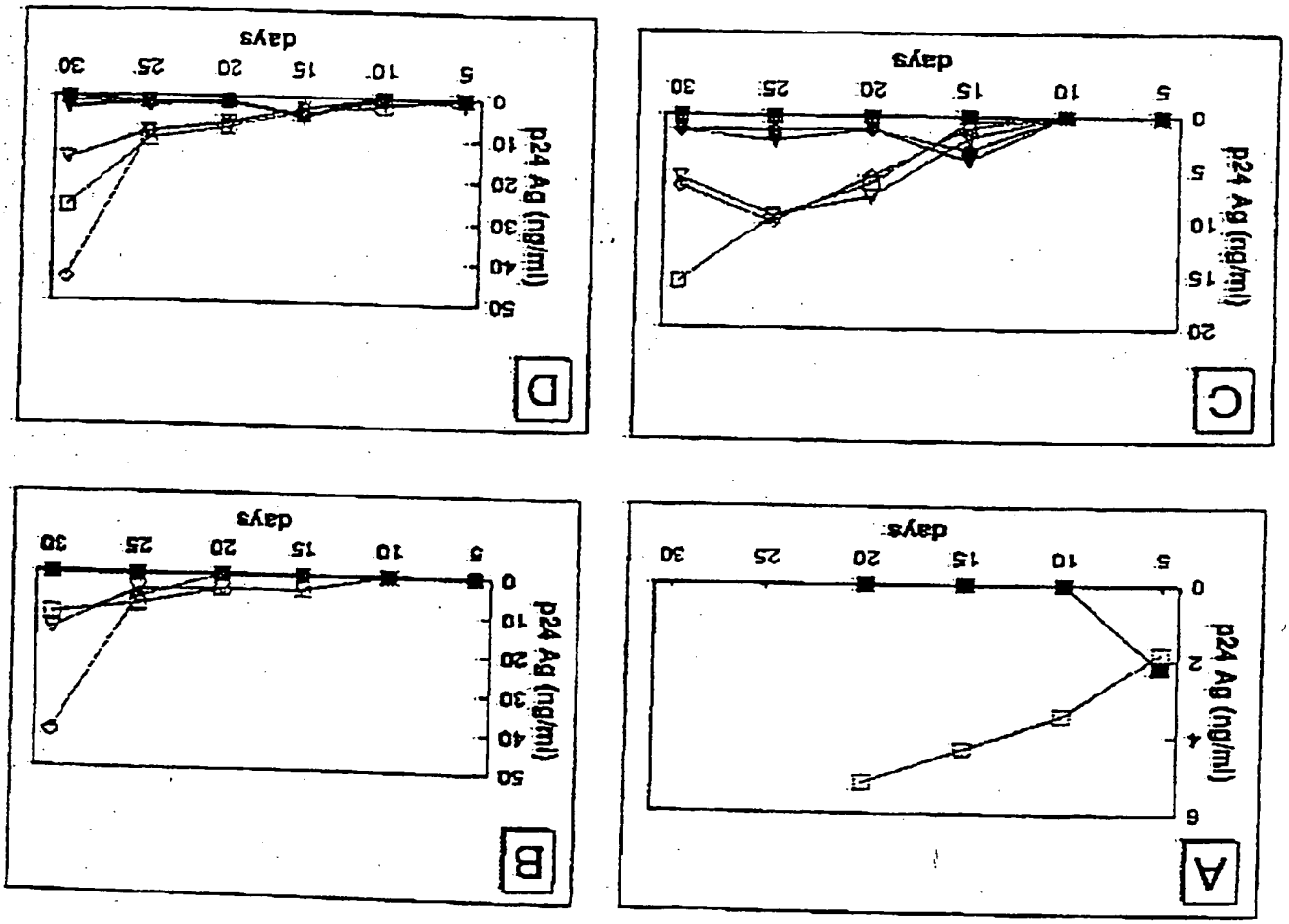

FIGURE 11 A, B, C, & D

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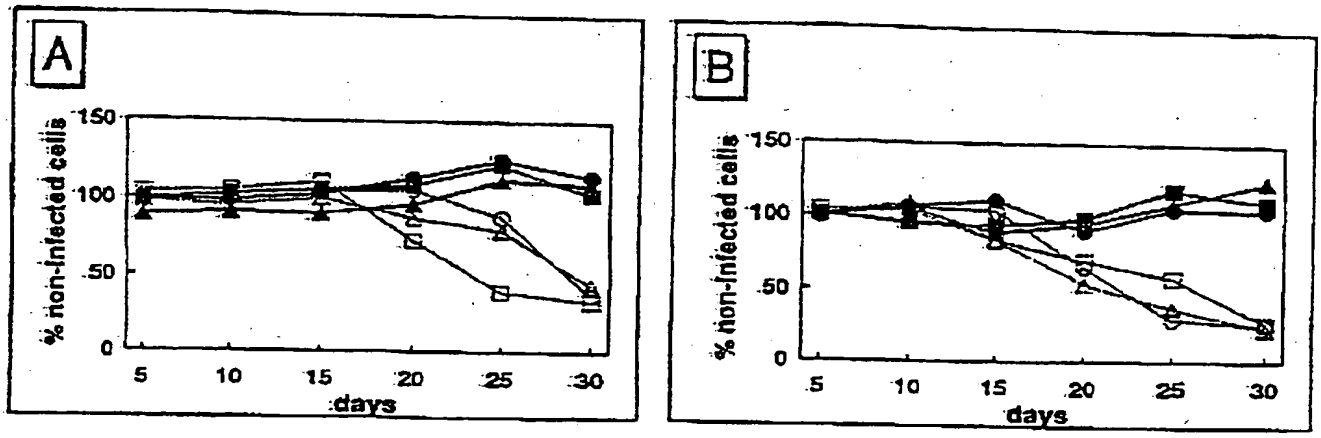
A
% non-infected cells
150
100
50
0
5
10
15
20
25
30
days
B
% non-infected cells
150
100
50
0
5
10
15
20
25
30
days

FIGURE 12 A & B

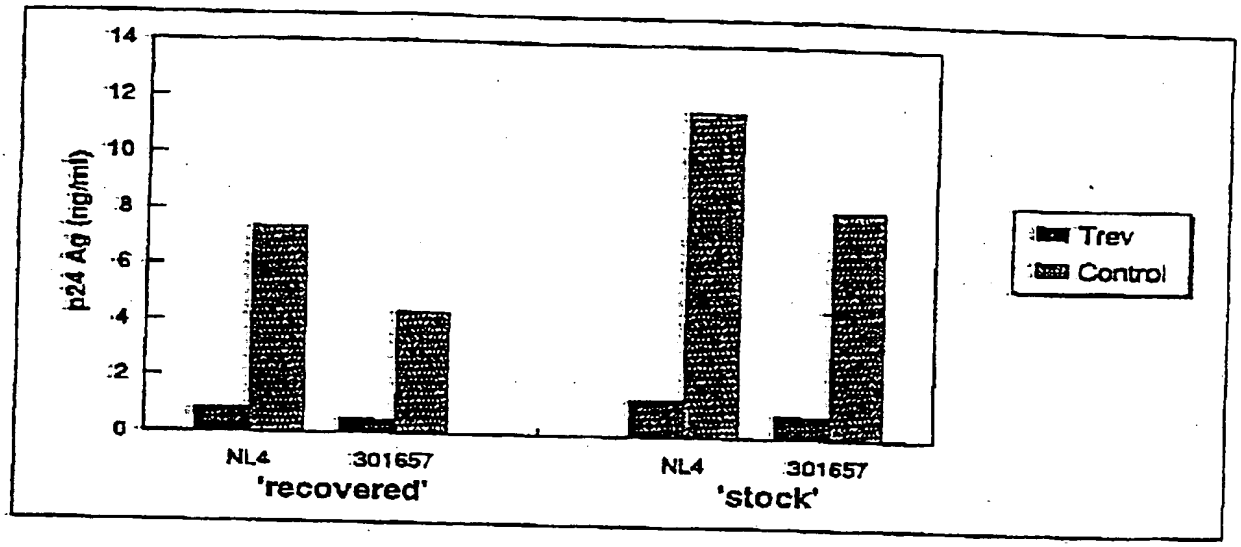

FIGURE 13

:DOCID: <WO___9628189A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02974

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 48/00; C12N 15/00

US CL : 514/44; 536/22.1, 23.1, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/22.1, 23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS,DIALOG (MEDICINE, BIOSCIENCES), STN (BIOSCIENCE)
fusion gene, fusion protein, transdominant, tat, rev

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOPE et al. trans-Dominant inhibition of human immunodeficiency virus type 1 rev occurs through formation of inactive protein complexes. Journal of Virology. 1992, Vol 66 pages 1849-1855 especially page 1850	1-7
Y	BOGERD et al. Journal of Virology. Dominant negative mutants of human T-cell leukemia virus type 1 rex and human immunodeficiency virus type 1 rev fail to multimerize in vivo. May 1993, Vol 67 pages 2496-2502, especially page 2499 and table 2.	1-7
Y	SOUTHGATE et al. Activation of transcription by HIV-1 Tat protein tethered to nascent RNA through another protein. Nature 14 June 1990, Vol 345 pages 640-642, especially page 640.	1-7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

25 JUNE 1996

Date of mailing of the international search report

22 AUG 1996

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Authorized officer

PATRICK TWOMEY, PH.D.

Telephone No. (703) 305-7022

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL [redacted]RCH REPORT

International [redacted]plication No.
PCT/US96/02974

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LIEM, et al. The development and testing of retroviral vectors expressing trans-dominant mutants of HIV-1 proteins to confer anti-HIV-1 resistance. Human Gene Therapy 1993, Vol 4 pages 625-634, especially figure 1 and page 632.	1-7
Y	GREEN et al. Mutational analysis of HIV-1 Tat minimal domain peptides: identification of trans-dominant mutants that supress HIV-LTR-driven gene expression. Cell 14 July 1989, Vol 58 pages 215-223, especially page 222.	1-7
Y	MALIM et al. Functional dissection of the HIV-1 Rev trans-activator - Derivation of a trans-dominant repressor of Rev function. Cell 1989, Vol 58 pages 205-214, especially figure 1.	1-7
Y	MALIM et al. Stable expression of transdomonant Rev protein in human T cells inhibits human immunodeficiency virus replication. Journal of Experimental Medicine October 1992, Vol 176 pages 1197-1201, especially page 1200.	1-7
Y	FEINBERG et al. Intracellular immunization: trans-dominant mutants of HIV gene products as tools for the study and interruption of viral replication. AIDS Research and Human Retroviruses 06 November 1992, Vol 8 pages 1013-1022, especially pages 1016-1017.	1-7
Y	PEARSON et al. A transdominant tat mutant that inhibits tat-induced gene expression from the human immunodeficiency virus long terminal repeat. Proceedings of the National Academy of Sciences. July 1990, Vol 87 pages 5079-5083, especially figure 1.	1-7

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